

# Distinct phenotypes distinguish the molecular classes of Angelman syndrome

A C Lossie, M M Whitney, D Amidon, H J Dong, P Chen, D Theriaque, A Hutson, R D Nicholls, R T Zori, C A Williams, D J Driscoll

R C Philips Unit and Division of Genetics, Department of Pediatrics, University of Florida, Gainesville, FL, USA

A C Lossie\*  
M M Whitney  
D Amidon  
H J Dong  
R T Zori  
C A Williams  
D J Driscoll

Center for Mammalian Genetics, University of Florida, Gainesville, FL, USA

A C Lossie  
D J Driscoll

Division of Biostatistics, Department of Statistics, University of Florida, Gainesville, FL, USA

P Chen  
A Hutson

GCRC, University of Florida, Gainesville, FL, USA  
D Theriaque  
A Hutson

Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania, Philadelphia, PA, USA  
R D Nicholls

Correspondence to: Dr Driscoll, Pediatric Genetics, Box 100296, University of Florida College of Medicine, Gainesville, FL 32610-0296, USA, driscjd@peds.ufl.edu

\*Present address: Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

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## Abstract

**Background—**Angelman syndrome (AS) is a severe neurobehavioural disorder caused by defects in the maternally derived imprinted domain located on 15q11-q13. Most patients acquire AS by one of five mechanisms: (1) a large interstitial deletion of 15q11-q13; (2) paternal uniparental disomy (UPD) of chromosome 15; (3) an imprinting defect (ID); (4) a mutation in the E3 ubiquitin protein ligase gene (*UBE3A*); or (5) unidentified mechanism(s). All classical patients from these classes exhibit four cardinal features, including severe developmental delay and/or mental retardation, profound speech impairment, a movement and balance disorder, and AS specific behaviour typified by an easily excitable personality with an inappropriately happy affect. In addition, patients can display other characteristics, including microcephaly, hypopigmentation, and seizures.

**Methods—**We restricted the present study to 104 patients (93 families) with a classical AS phenotype. All of our patients were evaluated for 22 clinical variables including growth parameters, acquisition of motor skills, and history of seizures. In addition, molecular and cytogenetic analyses were used to assign a molecular class (I-V) to each patient for genotype-phenotype correlations.

**Results—**In our patient repository, 22% of our families had normal DNA methylation analyses along 15q11-q13. Of these, 44% of sporadic patients had mutations within *UBE3A*, the largest percentage found to date. Our data indicate that the five molecular classes can be divided into four phenotypic groups: deletions, UPD and ID patients, *UBE3A* mutation patients, and subjects with unknown aetiology. Deletion patients are the most severely affected, while UPD and ID patients are the least. Differences in body mass index, head circumference, and seizure activity are the most pronounced among the classes.

**Conclusions—**Clinically, we were unable to distinguish between UPD and ID patients, suggesting that 15q11-q13 contains the only significant maternally expressed imprinted genes on chromosome 15.

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Keywords: Angelman syndrome; genotype-phenotype correlations; DNA methylation; 15q11-q13

Angelman syndrome (AS) (MIM 105830) is a severe neurobehavioural disorder that occurs with a frequency of ~1/15 000.<sup>1</sup> First documented by Dr Harry Angelman,<sup>2</sup> AS is now diagnosed by four cardinal features: (1) severe developmental delay; (2) profound speech impairment; (3) a movement and balance disorder; and (4) a characteristic behavioural profile that includes frequent, inappropriate laughter, a happy affect, and an easily excitable personality. Other common features include seizures, microcephaly, abnormal EEG patterns, sleep disturbances, hypopigmentation, and strabismus.<sup>3,4</sup>

Four major molecular mechanisms are known to cause Angelman syndrome. Most patients with AS (class I, deletion) have an ~4 Mb maternally derived interstitial deletion of 15q11-q13. Some patients (class II, UPD) have paternal uniparental disomy of the entire chromosome 15, while others (class III, ID) show defects in the imprinting process. In addition, intragenic mutations in the E6AP-E3 ubiquitin protein ligase gene (*UBE3A*) occur in several subjects with AS (class IV, *UBE3A*). Furthermore, a diagnosis of Angelman syndrome has been established in a large number of patients who have no known molecular lesion. These patients (class V) comprise a fifth group of subjects with AS. Although patients in classes I-III can be easily diagnosed by DNA methylation analyses along the 15q11-q13 imprinted domain,<sup>5</sup> subjects from classes IV and V show normal DNA methylation in this region.

The discovery of maternally inherited mutations in several patients with normal DNA methylation along 15q11-q13 first established a role for *UBE3A* in the aetiology of AS.<sup>6,7</sup> Subsequent studies identified an overall *UBE3A* mutation rate of 14-38% in sporadic patients.<sup>8-12</sup> Although cell lines from subjects with AS failed to show imprinted expression of *UBE3A*,<sup>13</sup> demonstration of brain specific imprinting by RT-PCR in the human confirmed the role of *UBE3A* in the pathogenesis of AS.<sup>14</sup>

Phenotypic analyses of several deletion (class I) patients have generated a profile considered by many to typify the classical AS phenotype. These patients showed a high incidence of severe, early onset seizures, microcephaly, and hypopigmentation.<sup>15,16</sup> However, patients from classes II and III often exhibited atypical features that ameliorated the classical phenotype. Patients with UPD and imprinting defects (ID) were less likely to present with

Table 1 BESS-T primers

Exon	Primer (5'→3')	Direction	Location	Size (bp)
6	TGCCAGCAGGTTTATTTT ACCAGCCTTGTGGTAAG	Forward Reverse	Exon Exon	187
7	GGAGAACCTCAGTCTGACGAC ATTGCGCTAGCTTCAATGTC	Forward Reverse	Exon Exon	38
8	GCAGCTGCAAAGCATCTAAT TCAATTCTAGCGCCTTCTT	Forward Reverse	Exon Exon	284
10*	GCAATCATCTTCTTTTCATGTT CGACACCATAATCATTAC	Forward Reverse	Intron Intron	194
11	TGTTACATACGATGAATCTACA CTCCCAAGTCACGAAAAGTT	Forward Reverse	Exon Exon	192
12	TCAGAGTTTAAAAGATTATTGGA TCCTGTTTTTCATTTGTAATTGG	Forward Reverse	Exon Exon	155
13	GAATTTGTCAATCTTTATTCTGACT CGGCTTCCACATATAAGCA	Forward Reverse	Exon Exon	155
14	AGATTTCCAAGCACTAGAAGAAA AATCAGAACAGAGTCCCTGG	Forward Reverse	Exon Exon	67
15	GTTCTGGGAAATCGTTTCATT CTGTGTCTGGGCCATTTT	Forward Reverse	Exon Exon	137
16*	ACCATGACTTACAGTTTTCCT TGGGACACTATCACCACCAA	Forward Reverse	Intron Intron	189

\*Kishino *et al.*<sup>5</sup>

hypopigmentation, microcephaly,<sup>17-21</sup> or seizures,<sup>18, 20, 21</sup> and often showed atypical features that were much milder than the deletion patients.<sup>18, 20, 21</sup> Analysis of 14 patients with mutations in *UBE3A* also suggested that class IV patients had a phenotype distinct from UPD and ID patients,<sup>22</sup> as they were more likely to present with microcephaly and seizures.

In the present study, we sought to conduct a large scale genotype-phenotype correlation of all five molecular classes in order to: (1) determine the accurate frequency of each molecular class; (2) provide detailed genotype based clinical data on each class; and (3) examine the molecular basis of the patients who have no known molecular defect. We report the highest *UBE3A* mutation frequency to date, show that

mutations in *UBE3A* do not account for all patients with normal DNA methylation along the AS/PWS imprinted domain, and examine alternative mechanisms for the aetiology of AS in class V patients. In addition, we present the first comprehensive analysis of genotype-phenotype correlations among all five molecular classes of AS and show significant differences among the five classes in growth parameters, achievement of developmental milestones, as well as severity, frequency, and onset of seizures.

## Materials and methods

### PATIENT REPOSITORY

#### Clinical definition of AS

Over the last 11 years, we have accumulated a large AS repository totalling 146 patients from 134 Angelman families. Each of our patients was extensively examined by clinical geneticists at the University of Florida (UF). Before laboratory testing, each patient was evaluated for 22 distinct criteria and given an AS rating of 1-5 based on clinical impression. We restricted this study to the 104/146 patients (93 families) who had an AS clinical score of 1 or 2. Patients who scored 1 showed an absolutely "classical" phenotype in every aspect. A score of 2 meant that the patient was fairly classical, but showed one or two features (for example, mild or absent seizures or obesity) that were atypical for AS. However, these patients were extremely likely to have AS, since they fulfilled all of the four main AS criteria,<sup>3, 4</sup> including the "AS specific" behaviour. The remaining 42 "AS-like" patients, all of whom had normal laboratory testing (DNA methylation, DNA polymorphism, and cytogenetic analyses) within 15q11-q13, exhibited some but not all of the four cardinal features of AS and were excluded from further investigation for the purposes of this study. Blood and tissue were procured with

Table 2 Sequencing primers

Exon	Primer (5'→3')	Direction	Location	Size (bp)
7	TATGGCCACCTGATCTGAC TTCCTATCTCCCATTACTGC	Forward Reverse	Intron Intron	345
8	GCTTGACTAACTTTTGCCTTG ATCTCCCACATGGTTTTCAG	Forward Reverse	Intron Intron	443
	GCAGCTGCAAAGCATCTAAT TCAATTCTAGCGCCTTCTT	Forward Reverse	Exon Exon	
9	AACAGCTATGACCATGCAACAGAGTAAACATACATATT* GTAAACGACGCGCCAGTCACTGAACGTATCAT*	Forward Reverse	Intron Intron	1401
	ATTAGGCCCTGATGATGTGT CCAGATATTCAGGACTGTGGA	Forward Reverse	Exon Exon	
10	TTGTTTCCTAATCCTACTCCTTG ACCAAATCCTTCTTTTGCTG	Forward Reverse	Intron Intron	352
11	GGACATTGTTTCTCAAGTGC TAAAAATGTCCCCCTTTGAG	Forward Reverse	Intron Intron	559
12	TGTTGTATTTGTAGTCTATGG† TTAATGAAGAGACAAAATGTGAC†	Forward Reverse	Intron Intron	257
13/14	GAAGTTCTTGTGATTAATGT† CCCTTTGGTGAATCAAATCTTCC†	Forward Reverse	Intron Intron	404
	GAATTTGTCAATCTTTATTCTGACT AATCAGAACAGAGTCCCTGG	Forward Reverse	Exon Exon	
15	TTCAGGATAAATTTGCTTGG AAAATCACGAATGTGCTCAG	Forward Reverse	Intron Intron	416

\*Matsuura *et al.*<sup>7</sup>†Malzac *et al.*<sup>8</sup>

Table 3 Molecular classes of Angelman syndrome

Class	Molecular defect	Families	%	Patients	%
I	15q11-13 deletion	63	68	64	61
II	Uniparental disomy (UPD)	7	7	7	7
III	Imprinting defect (ID)	3	3	7	7
IV	UBE3A mutation	10	11	15	14
V	Unknown	10	11	11	11

the approval of the families and the University of Florida Institutional Review Board.

#### Molecular diagnostics

Once a clinical diagnosis of AS was determined, the patient and family members were analysed by both molecular and cytogenetic tests in order to place each patient into the appropriate molecular class. Patients in classes I-III had uniparental DNA methylation at several loci along 15q11-q13, including *MKRN3* (formerly *ZNF127*), *PW71*, and 5' *SNURF-SNRPN*, while patients from classes IV and V showed normal biparental methylation at these loci.<sup>23-28</sup> DNA dosing,<sup>29</sup> high resolution chromosome and fluorescence in situ hybridisation (FISH) studies detected patients with large interstitial deletions (class I), while microsatellite and RFLP analyses separated patients in class II from those in class III.<sup>30-31</sup> Patients with normal DNA methylation were then tested for *UBE3A* mutations by a combination of Southern blot, BESS-T scan, and direct sequence analyses. Patients with intragenic *UBE3A* mutations were placed into class IV, while those with no detectable chromosome 15 abnormalities were put into class V.

#### Seizure criteria

According to our definitions, severe seizures occurred when at least two drugs were administered for epileptic episodes. When one anticonvulsant effectively controlled grand mal seizure activity, patients were considered to have moderate seizures. Mild seizures were defined by very rare grand mal, petit mal, or multiple febrile seizures. Patients with fewer than four febrile or a complete absence of seizures were considered to have no history of seizure activity.

#### SOUTHERN ANALYSIS

Southern blots were done according to standard conditions.<sup>32</sup> Patients were examined with genomic probes from the 5' end of *UBE3A*, as well as cDNA probes that covered exons 3-16. Genomic probes were generated by restriction digestion of cosmids 24 and 34.<sup>25</sup> cDNA probes were amplified using previously published primers for exons 3-9<sup>7</sup> and exons 9-16.<sup>6,7</sup> DNA methylation analyses of *UBE3A* were performed by hybridisation with a 2.7 kb *UBE3A* CpG island probe, which was generated by *EcoRI* to *NotI* digestion of cosmid 34.

#### METHYLATION SPECIFIC PCR

Sodium bisulphite treatment of genomic DNA and subsequent methylation analysis of *SNRPN* was done according to established protocols.<sup>32-33</sup> Two sets of primers were designed to assess the methylation status of both *NotI* sites in the *UBE3A* CpG island. At the proximal end of the CpG island (more 5'), the

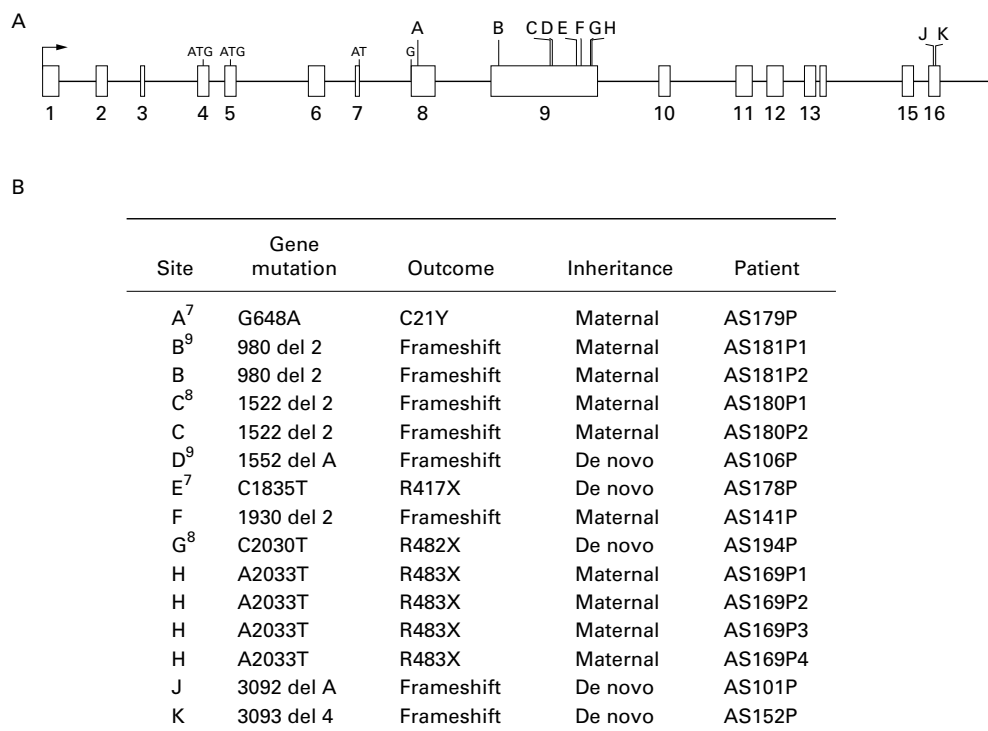
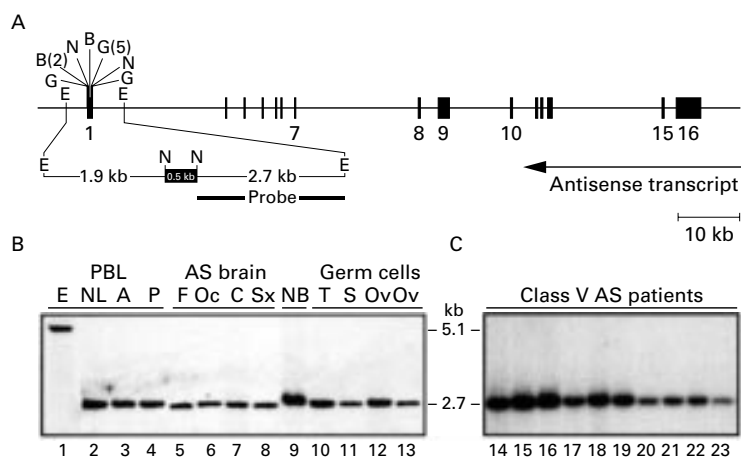


Figure 1 UBE3A mutations in patients from the UF repository. (A) Genomic organisation of *UBE3A*. *UBE3A* spans 120 kb of genomic DNA. The gene is shown to scale. Exons are numbered numerically from 1-16. Transcription (arrow) and translation (ATG) initiation sites are depicted. The primary translation initiation site begins in exon 8. The location of each mutation is indicated (A-K). (B) Description of mutations. The mutation from each family (A-K) is shown in column 2. Superscripts indicate previously identified mutations. Column 3 describes the predicted protein changes, while column 4 shows the inheritance of the mutation.



**Figure 2** DNA methylation analysis of 5' *UBE3A*. (A) Genomic structure of *UBE3A* and the 5' CpG island drawn to scale. Locations of selected restriction enzyme sites are depicted: *EcoRI* (E), *NotI* (N), *BssHIII* (B), *EagI* (G). Localisations of the 2.7 kb *NotI-EcoRI* probe as well as the 5.1 kb region containing the CpG island are indicated. The extent of overlap of the antisense transcript is also shown. If the CpG island is methylated, the 2.7 kb probe will hybridise to the 5.1 kb fragment on genomic DNA digested with *EcoRI* and *NotI*. If the CpG island is unmethylated, the probe will hybridise to itself, showing a 2.7 kb band on Southern blots. (B) Southern blot of normal (NL), AS (A), and PWS (P) genomic DNA from peripheral blood leucocytes (PBL); AS brain samples consisting of frontal cortex (F), occipital lobe (Oc), cerebellum (C), and coronal section (Sx); frontal cortex from normal brain (NB); adult testis (T); sperm (S); and fetal ovary (Ov) digested with *EcoRI* and *NotI*. DNA from normal PBL was also digested with *EcoRI* alone as a control (E). When digested with *EcoRI* and *NotI*, the 2.7 kb probe only hybridised to a 2.7 kb fragment, indicating that this site was unmethylated. (C) Southern blot analysis of PBL genomic DNA from class V patients digested with *EcoRI* and *NotI* shows that this site is unmethylated in all patients.

primers are as follows: methylated primer set (M1F 5'-CGTCGCGGTCGCGAGATT-3' and M1R 5'-ACGCGCCGAATCGACAAAA), unmethylated primer set (U1F 5'-GTG TTGTGGTTGTGAGATTTGTG-3' and U1R 5'-ACAAACACCCA CACTAACAATA ACT-3'). The primer sets that amplify the distal CpG island are as follows: methylated primer set (M2F 5'-GATAGGTAGCGGCGGTTGGC GACGAA-3' and M2R 5'-GCGCCTAAA CTACGACGACCGCCTCA-3'), unmethylated primer set (U2F 5'-GAATGTTG GGATTT TGGTGGT-3' and U2R 5'-AAACTACAA CAACCACCTCACTAATCA-3'). PCR products were stained with ethidium bromide and visualised on an Eagle Eye II photodocumentation system (Stratagene).

#### BESS-T SCAN

We analysed genomic DNA from patients following the procedures outlined in the BESS-T scan mutation detection kit (Epicentre, Madison, WI) using primers (table 1) corresponding to exons 6, 10, 11, 12, and 16. Cleaved products were size fractionated on 6% acrylamide, 6 mol/l urea and 30% (v/v) formamide sequencing gels according to standard protocols. Putative mutations were verified by sequence analysis.

#### SEQUENCE ANALYSIS

Abnormal PCR products from BESS-T scan analyses were cloned into TA vectors (Clontech) and sequenced using universal primers on an ABI 377 automated sequencer (ABI, Perkin Elmer). We designed coding region and intronic primers to amplify individual exons of *UBE3A* (table 2). PCR products were purified by the High Pure PCR Purification Kit

(Roche) and sequenced directly using a 1/4 reaction of the Big Dye terminators (ABI, Perkin Elmer). Reactions were purified by either repeated ethanol precipitation or by sepharose columns (Edge Scientific), dried under vacuum, resuspended, and fractionated on either an ABI 377 or ABI 373A automated sequencer. Sequence analysis was performed with the Sequencer program (Gene Codes Corporation).

#### PRIMERS

Collectively, primers for BESS-T (table 1) and direct sequence analysis (table 2) were designed to amplify the entire *UBE3A* coding region (exons 6-16). Novel primers were designed using the Primer 3 program from the Whitehead Institute.

#### STATISTICAL ANALYSES

Descriptive statistics were calculated as a percentage or by the mean (SD). All outcome variables were modelled as a function of age at evaluation, birth weight, molecular class (I, II, III, IV, and V), gestational age, maternal age, paternal age, race (white, Hispanic, and other), and sex. It should be noted that 93% of the UF patients and 56% of the total patients were white. Outcome variables consisted of continuous, binary, and ordinal measures, and were modelled using analysis of covariance (ANCOVA), logistic regression, and ordinal logistic regression, respectively. For binary and ordinal outcome variables with limited sample size, we employed the exact chi-square test. Odds ratios were used to summarise the binary and ordinal outcomes. All tests were two sided and considered significant at  $\alpha=0.05$ . All statistical analyses were performed using SAS 6.12 (SAS Institute, Cary, NC).

#### Results

##### MOLECULAR CLASSES OF ANGELMAN SYNDROME

Each patient was assigned a molecular class based upon results from high resolution chromosome, FISH, *UBE3A* mutation analysis, and DNA dosing, methylation, and polymorphism analyses along 15q11-q13 (table 3). The majority (63) of our classical families were deleted for the entire 4 Mb AS/PWS imprinted domain (class I). In seven cases, AS occurred via paternal UPD of chromosome 15 (class II), and three families had imprinting defects (class III). Intragenic mutations in *UBE3A* occurred in 10 of our classical patients (class IV), while the remaining 10 had no detectable chromosome 15 abnormalities (class V).

##### MUTATION ANALYSIS

Initially, a molecular confirmation of AS could not be made in 20/93 families using cytogenetic, DNA methylation, or DNA polymorphism tests. Hybridisation of 5' and 3' *UBE3A* cDNA probes to Southern blots did not detect genomic rearrangements in these patients (data not shown). However, sequence analysis of the *UBE3A* coding region in these 20 families showed intragenic mutations in 10 families (15 patients). We detected six deletions that resulted in frameshifts, three nonsense mutations, and



Table 4 Clinical and growth data

	<i>p</i> =		<i>Class I</i>		<i>Class II</i>		<i>Class III</i>		<i>Class IV</i>		<i>Class V</i>
	<i>UF</i>	<i>All</i>	<i>UF</i>		<i>UF</i>	<i>All</i>	<i>UF</i>	<i>All</i>	<i>UF</i>	<i>All</i>	<i>UF</i>
Speech	4.1×10 <sup>-3</sup>	2.0×10 <sup>-3</sup>	(21)		(7)	(27)	(7)	(20)	(15)	(46)	(11)
0 words			71	*	44	44	43	40	60	33	82
<3 words			29		43	15	14	15	27	26	9
3–10 words			*		43	33	43	30	13	41	9
>10 words			*		14	8	*	15	*	*	*
Seizure onset†	6.7×10 <sup>-2</sup>	7.6×10 <sup>-3</sup>	(20)		(3)	(14)	(2)	(9)	(12)	(24)	(5)
<1 y			10	*	7	7	*	33	17	13	40
1–2 y			40		33	14	*	23	8	4	20
2–3 y			25	*	14	14	50	11	25	29	40
3–4 y			25	*	*	*	*	*	50	29	*
>4 y			*		67	65	50	33	*	25	*
Seizure severity	2.0×10 <sup>-3</sup>	1.0×10 <sup>-3</sup>	(21)		(7)	(26)	(7)	(18)	(15)	(32)	(10)
Severe			29	*	4	4	14	6	33	19	10
Moderate			62		14	8	*	11	20	22	30
Mild			9		29	23	14	22	33	44	20
None			*		57	65	72	61	14	15	40
Height† (centile)	ND	2.9×10 <sup>-2</sup>	(21)		(7)	(28)	(7)	(17)	(15)	(30)	(9)
<5			42	*	7	7	30	12	7	10	*
5–20			5	*	11	11	14	12	20	10	56
21–40			14		43	18	14	18	46	27	22
41–60			19		14	14	14	23	13	33	*
61–80			10	*	18	18	14	23	7	17	22
81–95			10		14	21	14	6	7	3	*
>95			*		29	11	*	6	*	*	*
Weight (centile)	3.4×10 <sup>-3</sup>	3.7×10 <sup>-2</sup>	(21)		(7)	(27)	(7)	(15)	(15)	(28)	(9)
<5			18	*	4	4	*	*	*	*	11
5–20			14	*	7	7	29	13	13	7	33
21–40			24		14	4	*	*	*	*	11
41–60			24		14	15	*	*	27	14	34
61–80			10		14	15	29	13	13	18	*
81–95			*		14	22	*	20	27	18	*
>95			10		44	33	42	54	20	43	11
BMI (kg/m <sup>2</sup> ) (centile)	4.2×10 <sup>-3</sup>	1.7×10 <sup>-2</sup>	(21)		(7)	(17)	(7)	(14)	(13)	(13)	(9)
<5			10	*	*	*	*	*	8	8	11
5–20			10	*	*	*	*	*	8	8	33
21–40			24	*	6	6	*	*	*	*	*
41–60			18		14	6	*	*	8	8	34
61–80			18		14	12	57	29	31	31	11
81–95			10		29	29	*	7	14	14	11
>95			10		43	47	43	64	31	31	*
Head circum (centile)	3.2×10 <sup>-2</sup>	4.2×10 <sup>-2</sup>	(20)		(6)	(27)	(7)	(19)	(15)	(32)	(9)
<5			60	*	15	15	*	11	53	56	60
5–20			10		33	11	14	5	*	*	10
21–40			20		17	30	29	26	27	16	20
41–60			10		33	18	14	21	20	12	10
61–80			*		17	15	29	21	*	*	*
81–95			*		*	4	14	5	*	16	*
>95			*		*	7	*	11	*	*	*

Percentage of patients with each trait is indicated, with the sample size in parentheses.

ND = not determined.

UF = University of Florida data.

All = combined data from UF and published reports.

\*No patients in category.

†Statistically significant in combined data set only.

All other parameters are statistically significant in UF and combined data sets.

one missense mutation (fig 1). We found a 50% mutation detection rate with 3/4 familial (75%) and 7/16 sporadic cases (44%) containing mutations within the coding region of *UBE3A*. In our examination of published reports, we found that six of our patients had lesions in *UBE3A* that were identical with previously published mutation sites. Further studies indicated that these sites were not mutation hotspots, but independent reports of mutations in patients A, B, C, D, E, and G.<sup>7-9</sup>

In addition to disease associated mutations, we also found three novel polymorphisms. A maternally inherited silent transition (A1144G) occurred in exon 9 in one subject. No other mutations were found in this patient. A common polymorphism was detected in a poly T tract within intron 6. Sequence analysis of 67 subjects showed that 17% of the chromosomes ( $p=0.83$ ,  $q=0.17$ ) contained a 1 bp thymidine insertion at

nucleotide position -47, relative to exon 7. One AS-like patient had a paternally inherited 14 bp deletion of the 3' UTR. No disease associated mutation was found in this person. In addition, BESS-T scan detected one subject who was homozygous for a previously identified expressed polymorphism in exon 6.<sup>34</sup>

#### DNA METHYLATION OF 5' *UBE3A*

Although we identified disease causing mutations in 50% of our patients who had normal DNA methylation along 15q11-q13, the etiology of AS in the remainder of our classical Angelman syndrome patients was unknown. We postulated that disruption of the normal *UBE3A* DNA methylation pattern in class V patients could be causative of AS in these patients.

Using rare cutting restriction enzymes, we isolated a 2.7 kb *NotI-EcoRI* fragment from the CpG island at 5' *UBE3A* to test for parent of



Table 6 Clinical data II

	<i>p</i> =		Class I		Class II		Class III				Class IV				Class V	
	UF	All	UF	(n)	UF	(n)	All	(n)	UF	(n)	All	(n)	UF	(n)	All	(n)
Walk by 5 y*	2.9×10 <sup>-3</sup>	1.1×10 <sup>-5</sup>	50	(20)	100	(7)	96	(25)	86	(7)	94	(18)	93	(15)	97	(35)
Feeding problems (NS)	0.6×10 <sup>-1</sup>	1.2×10 <sup>-1</sup>	70	(20)	80	(5)	75	(12)	100	(5)	83	(18)	80	(10)	46	(24)
Simple commands*	3.1×10 <sup>-3</sup>	1.2×10 <sup>-3</sup>	53	(17)	100	(4)	91	(11)	100	(7)	100	(13)	100	(13)	100	(13)
Seizures*	6.3×10 <sup>-4</sup>	6.8×10 <sup>-4</sup>	100	(21)	43	(7)	45	(31)	29	(7)	59	(27)	53	(15)	73	(48)
Significant seizures*	1.0×10 <sup>-4</sup>	8.3×10 <sup>-8</sup>	90	(21)	14	(7)	12	(26)	14	(7)	17	(18)	53	(15)	41	(41)
Hypopigmented*	3.3×10 <sup>-7</sup>	2.8×10 <sup>-8</sup>	90	(21)	17	(6)	20	(20)	0	(7)	14	(22)	15	(13)	23	(43)

Percentages of patients with each phenotype are indicated under category, with the sample size shown in parentheses.

UF = University of Florida set only.

All = combined data from UF and published reports.

\*Significant in both data sets.

NS = not significant.

anticonvulsant) occurred in 19/21 deletion patients, but only in 1/7 UPD, 1/7 ID, 8/15 *UBE3A* mutation, and 4/10 class V patients ( $p=9.96 \times 10^{-3}$ ). Similar findings were observed in the combined data set (table 6,  $p=8.26 \times 10^{-8}$ ). In addition, 90% of class I patients were hypopigmented compared to family members (table 6,  $p=3.33 \times 10^{-8}$ ). However, less than 25% of classes II-IV and 44% of patients in class V showed evidence of hypopigmentation.

## Discussion

### MOLECULAR BASIS OF AS IN CLASSES IV AND V

Molecular analyses showed that 20 of the 104 classical patients with AS (93 families) we studied had normal DNA methylation at several imprinted 15q11-q13 loci, excluding large deletions, UPD, or imprinting defects from the aetiologies of AS in these subjects. We found mutations in 50% of these patients, with

44% (7/16) of our sporadic and 75% (3/4) of our familial cases having mutations within *UBE3A*. Recent mutation analyses by other laboratories showed a lower *UBE3A* mutation frequency (5-38%) in their sporadic patients.<sup>8-12 57</sup>

Including our patients, *UBE3A* mutations have been identified in 51 AS families.<sup>8 12 54 57</sup> Forty-five are unique mutations (fig 4), with no more than three unrelated subjects sharing a common error. Most defects are nonsense mutations that are predicted to result in premature termination during translation, and therefore may not represent true null mutations. However, the last six amino acids of *UBE3A* are crucial for normal protein function in the presence of human papillomavirus,<sup>58</sup> and it is possible that these mutations produce unstable message owing to nonsense mediated decay.<sup>59 60</sup> Although defects have been found in

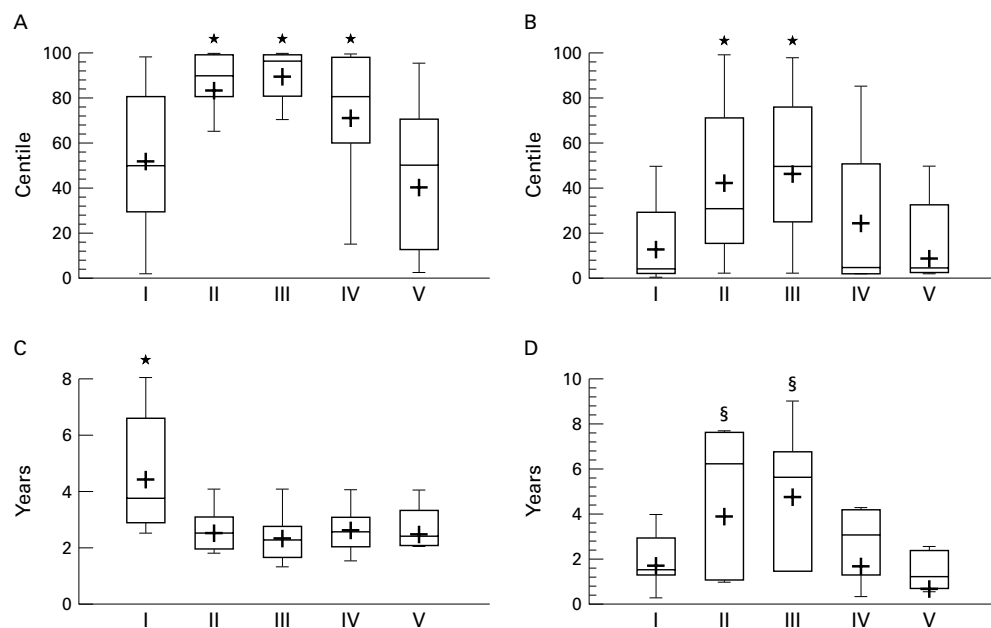


Figure 3 Mean BMI, HC, age to walk, and onset of seizures. Boxes show the extent of 25-75 % of the patients, while bars (T) depict the range. The medians (-) and means (+) for each class are shown. Phenotypes that are statistically significant in the UF and combined data sets (\*) or in the UF data only (§) are indicated. Molecular class (I-V) is shown at the bottom of each graph. The centiles (A and B) and age in years (C and D) are shown on the left. (A) Body mass index percentiles. Statistically, patients in classes II, III, and IV have higher BMI percentages than patients in classes I and V (B) Head circumference percentiles. Patients in classes II and III are statistically different from patients in class I, but not from patients in classes IV and V. There are no statistically significant differences between classes I, IV, and V. (C) Mean age to walk. Statistically significant differences are observed in patients in class I compared with the other four classes. (D) Mean age at onset of seizures. In the UF data set, seizures, when they occur, start much later in classes II and III than the other classes.

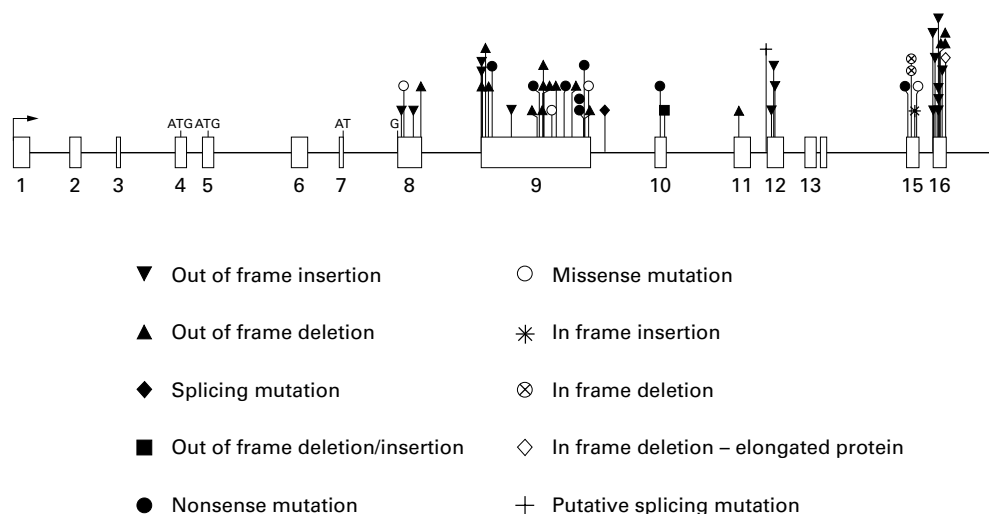


Figure 4 Summary of all published mutations in *UBE3A*. The gene is shown to scale. Exons are numbered numerically from 1-16. Transcription (arrow) and translation (ATG) start sites are noted. The primary translation initiation site begins in exon 8. The location of each mutation is indicated by a symbol. Filled symbols indicate protein truncating mutations. Symbols are stacked at positions where two unrelated subjects share a common mutation. There are 51 total mutations in unrelated subjects. Forty-five mutations are unique. Familial mutations are counted once. Most mutations (38/45) are protein truncating and cluster in exons 9 and 16.

virtually all protein coding exons, most cluster in exons 9 and 16 (fig 4). Exon 16 is within the HECT domain, a region of high conservation between different E3 protein ligases.

Although our study has shown the highest *UBE3A* mutation frequency found to date in sporadic cases, our results indicate that approximately 11% of patients with AS have an unknown aetiology. Alternative explanations must account for the molecular basis of AS in this group. Five possible explanations follow. (1) Many *UBE3A* mutations occur in non-coding regions; (2) *UBE3A* can be inactivated by other mechanisms; (3) other genes, some of which may reside in the ubiquitin pathway (genome wide), can also cause AS; (4) AS associated mutations can occur in other 15q11-q13 genes; (5) these patients do not have AS, but instead have disorders that mimic AS.<sup>61</sup> However, our rigorous clinical criteria and extensive clinical expertise with AS suggest that the last explanation is unlikely.

Although the relative lack of *UBE3A* mutations in patients with normal DNA methylation could be accounted for if various mutations occurred outside the *UBE3A* coding region, we did not detect genomic rearrangements in any of our 20 families, and only one genomic rearrangement has been identified to date.<sup>53</sup> Furthermore, no mutations have been reported in the promoter region or 3' UTR of *UBE3A*.

We postulated that disruption of the normal DNA methylation pattern at 5' *UBE3A* could inactivate the gene. With the exception of only a few genes, the promoter regions of most imprinted genes show differential methylation based on the parental origin of the chromosome.<sup>62-63</sup> In the AS/PWS domain, several paternal only expressed genes display parent of origin DNA methylation imprints, with the promoters of the active allele being hypomethylated compared to the inactive allele.<sup>24-26 64-66</sup> Since DNA methylation of promoter regions is closely associated with transcriptional repression,<sup>68</sup> we hypothesised that methylation of the

maternally derived 5' CpG island of *UBE3A* could repress imprinted expression in class V patients. In addition, recent experiments suggested that in somatic cell hybrid cell lines, which are derived from skin fibroblasts, *UBE3A* was methylated on the maternal allele.<sup>67</sup> However, in contrast to Meguro *et al*,<sup>67</sup> we found that both parental alleles of *UBE3A* were completely unmethylated in PBL, skin fibroblasts, germ cells, and brain, which has been shown to demonstrate imprinted expression of *UBE3A*.<sup>14</sup> In addition, class V patients were completely unmethylated at 5' *UBE3A* in PBL DNA, indicating that they probably do not inactivate *UBE3A* by inappropriate methylation. To eliminate this possibility fully, brain DNA from class V patients should be examined.

Our data suggest that disruption of *UBE3A* may not be the only cause of AS and other factors may also be involved. The most obvious candidates are other genes in the ubiquitin pathway. Any protein that regulates or interacts with *UBE3A* in this cascade and all proteins that *UBE3A* targets for degradation are candidates for patients grouped in class V. So far, four proteins have been identified as substrates for *UBE3A* mediated degradation: p53, HHR23A, the Src family kinase Blk, and *UBE3A* itself.<sup>69-74</sup>

Alternatively, other 15q11-q13 genes may play important roles in the aetiology of class V patients. The *UBE3A* antisense transcript is one candidate, as preliminary data suggest that the paternally expressed antisense transcript is restricted to the brain.<sup>75</sup> It is possible that expression of the antisense transcript from the paternal chromosome specifically inactivates the paternal *UBE3A* allele in *cis*. According to this model, biallelic activation of the antisense transcript would result in repression of both *UBE3A* alleles. It would be important to know if class V patients show biallelic expression of the *UBE3A* antisense transcript in the regions of the brain that shows imprinted expression of



*UBE3A*. Another excellent candidate gene is the newly identified imprinted gene *ATP10C*. This gene is located approximately 200 kb distal to *UBE3A* and is preferentially expressed from the maternal allele in human brain.<sup>76 77</sup> It would be interesting to determine if some of our class V patients have lesions within this new candidate gene.

#### GENOTYPE-PHENOTYPE CORRELATIONS

The second major goal of this study was to establish phenotypic profiles for each molecular class. Genotype-phenotype analyses of our patients indicated that all five classes had the four cardinal features of AS (that is, severe developmental delay, profound speech impairment, a movement and balance disorder, and characteristic behaviour). Statistical models showed that the five classes could be separated into four phenotypic groups: class I, classes II and III, class IV, and class V. The statistically significant clinical distinctions between the classes include 16 of 22 parameters ( $p < 0.05$ ), and were most striking for pigmentation, growth parameters, achievement of developmental milestones, and severity, frequency, and age of onset of seizures.

We found that the class I (deletion) patients as a group have the most classical and severe phenotype. They achieve developmental milestones later and to a lesser degree than the other classes. Statistical models show that deletion patients learn to sit and walk later than the other classes and are less likely to be able to follow simple commands. Class I patients have the highest incidence of severe seizures (90%) and hypopigmentation (90%) of all five classes. In addition, deletion patients typically have a complete absence of speech, a normal BMI distribution, and severe microcephaly.

In contrast, UPD and ID patients (whom we found as a group to have indistinguishable phenotypes) are much less severely affected. They have a low incidence of hypopigmentation, microcephaly, and severe seizures. In addition, almost half of UPD and ID patients have more than three words in their vocabulary. Patients in classes II and III are larger and heavier than class I patients. Analysis of body weight by BMI showed that more than 70% of these patients are above the 80th centile for their age group. Our data from these two classes correlate well with the combined data of others who also found fewer incidences of hypopigmentation, microcephaly, or seizures in UPD and ID patients.<sup>17-21 41 45</sup>

*UBE3A* mutation patients fall somewhere in the middle. They are statistically similar to deletion patients with respect to seizures, absence of speech, and microcephaly. Moncla et al<sup>22</sup> examined class IV patients individually for head circumference and seizures, and similarly concluded that *UBE3A* mutation patients had high incidences of seizures and microcephaly. Statistically, the class IV patients that we examined are similar to UPD and ID patients in the development of motor skills, ability to follow simple commands, pigmentation, and development of obesity. Class IV patients have a high incidence of early onset

obesity. Perhaps *UBE3A* functions in the same pathway as the newly discovered *ATP10C* gene, whereby lesions in *UBE3A* also alter expression of *ATP10C*. In addition, *UBE3A* mutation patients differ from UPD and ID patients in that they were more likely to have a history of moderate to severe seizures and microcephaly. Although milder than deletion patients, class IV cases have a more typical phenotype than class II or III patients, indicating that lesions in *UBE3A* have pleiotropic consequences.

Class V patients are very interesting. Although these patients have no detectable chromosome 15 abnormalities, statistically they are most similar to the deletion patients. Class V patients have normal height, weight, and BMI distributions, similar to those observed in class I patients. They also frequently present with a complete absence of speech, early onset of seizures, hypopigmentation, and microcephaly. The observation of hypopigmentation in these patients is unexpected. One possible explanation could be that the hypopigmented subjects are haploinsufficient for the *P* gene or for another pigmentation gene located elsewhere in the genome. However, class V patients differ from deletion patients in two important characteristics. They walk about two years earlier than deletion patients and they are less likely to have seizures compared to patients in class I.

In addition, class V patients tend to have a more severe phenotype than that observed in classes II, III, and IV. It is possible that disruption of more than one gene is causative of the class V phenotype in these patients. Perhaps these patients have mutations in a gene or "controlling region" in 15q11-q13 that affects additional loci within the domain, such as *ATP10C*, giving rise to the more severe phenotype. Alternatively, these patients may have defects upstream of *UBE3A* that alter additional genes within the ubiquitin pathway. However, some class V patients could suffer from a loss of function mutation in a *UBE3A* target protein. This could lead to a more severe phenotype than that observed in *UBE3A* mutation patients, who presumably experience an upregulation of a critical *UBE3A* target protein. In addition, two recent papers have shown a link between "AS-like" subjects and *MECP2*.<sup>78 79</sup> Although at least one of our "AS-like" patients was later diagnosed with Rett syndrome, it remains to be seen if any of our class V patients have mutations in *MECP2*, the gene responsible for Rett syndrome.

Our data indicate that class I patients comprise a contiguous gene disorder, with haploinsufficiency of additional non-imprinted genes exacerbating the lack of the maternally expressed *UBE3A* gene. For example, hypopigmentation has been linked to haploinsufficiency of the *P* gene in PWS deletion patients,<sup>80</sup> and the inheritance of a single copy of the *GABRB3* gene has been implicated in seizure susceptibility.<sup>81</sup> Our data clearly show that patients in classes II and III have the least severe and most atypical phenotype, suggesting that a "double dose" of the paternal only

expressed genes may ameliorate the AS phenotype in these patients. In addition, the finding that class II and III patients are larger and have better motor skills than the other patients with AS may have implications for the "parental conflict" hypothesis, which states that paternally expressed genes are involved in embryonic or postnatal growth.<sup>82</sup> For our findings to support this hypothesis, it will be important to establish if the increased growth and obesity observed in UPD and ID patients occurs while the offspring is still capable of using maternal resources (for example, breast feeding).

Another important finding is that almost half of the *UBE3A* mutation patients and 70-75% of the UPD/ID patients have extremely high BMI ratios (above the 80th centile). Obesity is also observed in several mouse models of AS. Mice that have paternal UPD for the AS orthologous region,<sup>83</sup> a 4 Mb maternally derived deletion of the AS/PWS region<sup>84</sup> (Nicholls, unpublished data), or a smaller maternally derived deletion in this region,<sup>85-86</sup> have adult onset obesity. Genetic studies in the mouse have mapped a maternally expressed "fat" gene (*pfatp*) very close to the *UBE3A* gene.<sup>85</sup> The human orthologue, *ATP10C*, shows imprinted expression in human brain and maps very close to *UBE3A*.<sup>76-77</sup> An integrated hypothesis would suggest that *UBE3A* and/or another closely linked gene (that is, *ATP10C*) play a role in metabolism and/or feeding behaviour. The relative lack of obesity in class I patients could be caused by the severe underlying motor defects associated with deletion patients.

This comprehensive and extensive study of genotype-phenotype correlations provides additional delineation and enhances our understanding of the distinct clinical subgroups of AS. Our data indicate that patients with a high body mass index and a history of significant seizures would be good candidates for *UBE3A* mutation analysis. It is clear that although mutations in *UBE3A* are sufficient to cause the four cardinal features of Angelman syndrome, as well as recurrent seizures and microcephaly, other 15q11-q13 genes must contribute to the development of motor skills, severity of seizures, cognition, growth, and obesity found in the other classes of AS. Elucidating the role of these other 15q11-q13 genes will give us important insights into mammalian development and into an improved understanding of contiguous gene deletion disorders. Delineating all the *UBE3A* target proteins could promote the design of rational therapies for AS. Furthermore, discovery of the molecular mechanism(s) involved in the pathogenesis of class V patients will be crucial to our full understanding of Angelman syndrome and the molecular complexity governing phenotypic expression of imprinted genes.

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